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Studies Supporting the Refinement and Validation of a PBPK Model for 1,4-Dioxane: In Vitro Metabolism in Cryopreserved Human Hepatocytes

Poet, T.S., Creim, J.A., Soelberg, J.J., Thrall, K.D., and Corley, R.A.

Submitted To:

Betty Locey, Ph.D., DABT
Arcadis G&M of Michigan, LLC
25200 Telegraph Rd.
Southfield, MI 48034
blocey@arcadis-us.com

Submitted By:

Battelle, Pacific Northwest Division
Center for Biological Monitoring & Modeling
902 Battelle Blvd.
P.O. Box 999, MS P7-59
Richland, WA 99352

Corresponding Author:

Torka S. Poet, Ph.D.
Ph: 509-375-7740
Fax: 509-376-9064
e-mail: Torka.poet@pnl.gov

ABSTRACT

1,4-Dioxane (dioxane) is a colorless liquid widely used for its solvent properties. A physiologically based pharmacokinetic model (PBPK) is under development to describe the disposition of dioxane in rats, mice, and humans. The metabolism of dioxane to β-hydroxyethoxyacetic acid (HEAA) was determined in cryopreserved isolated hepatocytes from human donors incubated in individual vials for 60 minutes with varying initial concentrations of dioxane. Michaelis-Menten rate constants (K_m and V_{max}) were determined using non-linear kinetic analysis. The Michaelis-Menten constant, K_m, was 7.7 $\pm$ 8.4 mg/ml and the V_{max} was 4.8 $\pm$ 3.1 μg/hr/10^6 cells. Michaelis-Menten rate constants for the metabolism of dioxane in rats and mice have previously been reported. The Michaelis constant in rat and mouse hepatocytes was 2.5 and 2.6 mg/ml, respectively. The V_{max} in these species were nominally lower than in human hepatocytes (1.92 and 3.74 μg/hr$\times 10^6$ cells for rats and mice, respectively).

INTRODUCTION

1,4-Dioxane (dioxane) is a colorless liquid that has been used as a solvent or as a stabilizer in solvents. Dioxane is postulated to be primarily metabolized by cytochromes P450 to *p*-dioxane-2-one and β-hydroxyethoxyacetic acid (HEAA), depending on the pH of the analytical methods (Woo et al., 1977; Braun and Young 1977). Two physiologically based pharmacokinetic (PBPK) models have been developed to describe the disposition of dioxane in rats and humans (Leung and Paustenbach, 1990; Reitz et al., 1990) with the latter model extended to mice. Although both models utilized similar data sets in their development, there were notable differences in the derivation of partition coefficients and metabolic rate constants. Therefore, the purpose of the study described herein was to determine *in vitro* metabolic rate constants for dioxane in cryopreserved human hepatocytes. This report is an expansion on a previous report (Poet et al., 2006), where experimental methods are described in more detail.

METHODS AND MATERIALS

*Chemicals***.** 1,4-dioxane was obtained from Aldrich Chemical Co. (St. Louis, MO). HEAA was a gift from Dow Chemical (Midland, MI).

Isolated Hepatocytes. Cryopreserved isolated hepatocytes from male human donors were purchased from In Vitro Technologies (Baltimore, MD). Cell viability (membrane integrity) was assessed by trypan blue dye exclusion. Male human donor demographics, as provided by the supplier, are given in Table 1. Pilot

studies were conducted to optimize the final substrate concentrations and time course for full studies. For the final incubations, targets of 2.25 $\times 10^6$ cells/ml in 0.25 ml Krebs-Henseleit bicarbonate buffer (pH 7.4) with 12.5 mM Hepes were incubated with 6 concentrations of dioxane (0.25 - 50 mg/ml Dioxane) at 37°C for 60 min in a shaking water bath. The reaction was stopped by the addition of 0.25 ml of a 1.0 N HCl/internal standard (glycolic acid) solution. The production of HEAA in these incubations was measured (see Analytical Methods below) and evaluated based on Michaelis-Menten kinetics. Some additional samples were incubated with 0-25 mg/ml dioxane for 30 min to determine cell count/viability after incubation.

Analytical Methods. To measure HEAA, the hepatocyte samples were extracted twice using 2 ml methyl t-butyl ether containing tri-n-octylphosphine oxide. The supernatant was evaporated to dryness under an ultra high purity nitrogen stream and reconstituted with 0.1 ml toluene and 50 µl n-methyl-n-tert-butyldimethylsilyl-trifluoroacetimide (MTBSTFA) as a derivatizing agent and incubated for 1 hr at 60ºC. HEAA concentrations were determined using a GC/MS method on an Agilent Model 6890 GC (Avondale, PA) with a mass selective detector (model 5973N). The column was a Restek (Bellefonte, PA) Rtx-5MS (30 m x 0.25 mm id x 0.25 µm df). The GC oven was programmed to ramp from an initial 40ºC to a final temperature of 280ºC at a rate of 18ºC/min. The temperatures of the injection port and MS interface were 200ºC and 280ºC, respectively. Quantitation was achieved using m/z 247 for glycolic acid as the internal standard with a retention time of approximately 8 min and m/z 291 for

HEAA, with a retention time of approximately 9.9 min. All ions were acquired in scan mode. The limit of reliable quantitation was 0.25 µg/g.

RESULTS AND DISCUSSION

The goal of this research was to assess and compare the *in vitro* metabolism of dioxane in mouse, rat, and human cryopreserved hepatocytes to aid in the refinement of a PBPK model for 1,4-dioxane. The results and metabolic rate constants for rats and mice have been reported previously (Poet et al., 2006).

Cryopreserved hepatocytes have recently become a viable option for human hepatocyte metabolism studies since they are easier to obtain and use in suspension cultures than fresh hepatocytes and studies have demonstrated the validity of metabolic parameters determined in these cell preparations (Li *et al.,* 1999; Hengstler *et al.,* 2000; Hewitt *et al.,* 2001; Naritomi *et al.,* 2003). Due to the expense associated with obtaining hepatocytes from human donors compared to rat or mouse hepatocytes, pilot studies exploited the results from the previous animals as starting points for numbers of cells and dioxane concentrations that would be utilized in human in vitro studies.

Pilot incubations at initial dioxane concentrations of 0.5 and 25 mg/ml dioxane in a target cell density of 2.5×10^6 cells/ml in 250 µl demonstrated a linear production of HEAA from both concentrations over 90 min. The increased production of HEAA between these two dioxane concentrations was less than proportional to the starting concentrations of 1,4-dioxane, suggesting that the Km would likely be below 10 mg/ml in the definitive studies (Fig. 1).

Based on the results of the preliminary studies, hepatocyte incubations were carried out at initial dioxane concentrations ranging from 0.25 to 50 mg/ml for 60 minutes at a target cell density of 2.5×10^6 cells/ml. HEAA concentrations were normalized to 1×10^6 cells/ml (Appendix A and B) and *in vitro* Michaelis-Menten rate constants determined using the analyze function of GraphPad Prism (San Diego, CA).

Selected samples from donors CEC and EQB were incubated with dioxane for 60 min and trypan blue dye exclusion used as a measure of cell membrane integrity (Fig. 3). Initially, cells were incubated with the dioxane concentrations used in the metabolism study design, but it was determined that viability in the samples incubated with 10 mg/ml dioxane or less was not affected, whereas cells incubated with 25 mg/kg showed appreciably more leakage of the die through the membrane. The pilot time linearity studies were conducted using 25 mg/ml dioxane and indicate linear HEAA production in these samples for at least 90 minutes, suggesting that the apparent affects on cell membrane do not adversely affect dioxane metabolism at least through 90 min. However, HEAA production in cells incubated with 50 mg/ml dioxane tended to be lower than in samples incubated with 25 mg/ml dioxane. The 50 mg/ml dioxane samples were therefore not included in the determination of dioxane Michaelis-Menten rates.

The reported major metabolite of dioxane is p-dioxane-2-one. The analytical methods used in this study results in the conversion of p-dioxane-2-one to HEAA, and the V_{max}/K_m reported is for the cumulative transformation of dioxane to both potential products, as measured by the production of HEAA. The V_{max} for the

production of HEAA from dioxane ranged from 2.4 to 8.7 μg/hr/10^6 Cells, and the Km ranged from 3.8 to 17.6 mg/ml (Table 2; Fig. 2). The rate constants and metabolite profile from three of the human donor hepatocytes samples are very similar, and the results from donor, sample EQB, indicate a possible outlier with a higher Km (Table 2).

The supplier (In Vitro Technologies) provided marker substrate metabolism data, and these values were compared to the Vmax and pseudo-first order rate (V_{max}/K_m) for dioxane. Correlations greater than 50% were only obtained between the reported 7-hydroxylation of coumarin (Fig. 4) and O-demethylation of dextromethorphan (data not shown). The correlation between the pseudo-first order rate for dioxane metabolism and the metabolism of coumarin was good (r^2=0.83), the correlation for dextromethorphan was not nearly as good (r^2=0.63). Coumarin is metabolized by a number of CYP450 enzymes, but the principle P450 is 2A6. Dextromethorphan is primarily metabolized by CYP450s from the 2D family. Adding (or averaging) the metabolism of coumarin and dextromethorphan results in a slight increase in the correlation with the pseudo-first order rate constant to r^2=0.86. This suggests a possible relationship between the combined activities P450s 2A and 2D and the metabolism of dioxane.

The *in vitro* Michaelis-Menten rate constants in human isolated hepatocytes are remarkably similar to the values obtained in rat and mouse hepatocytes (Poet et al., 2006). The Michaelis-Menten constant in rat and mouse hepatocytes was 2.5 and 2.6 mg/ml, respectively. The V_{max} in these species were nominally lower than

in human hepatocytes (averages of 4.8, 1.9 and 3.7 μg/hr$\times 10^6$ cells for human donors, rats, and mice, respectively).

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Table 1. Human donor demographics and cell conditions.

Lot	Age	Viability[1]	Cause of Death	Tobacco Use[2]	Alcohol Use	Substance Use	Medical History
CEC	48	86%	Intracranial hemorrhage	Yes	Yes	THC	Rheumatoid arthritis
EQB	72	88%	Intracranial hemorrhage	Yes	No	No	Prostate remove
HHG	58	83%	Cerebrovascular accident	Yes	No	No	High Cholesterol
VCM	68	82%	Head Trauma	No	Yes	No	No
BEW[3]	NA	75%	NA	NA	NA	NA	NA

[1] Viability reported by the supplier.

[2] No – None reported

[3] Pool

Table 2. Rate constants for the saturable metabolism of dioxane to HEAA in hepatocytes from human donors.

	CEC	EQB	HHG	VCM	BEW	AVG[1]	SD	AVG[2]	SD
Vmax (μg/hr/10^6 Cells)	4.32	8.74	2.44	3.45	5.93	4.84	3.11	3.40	0.94
Km (mg/ml)	4.11	17.6	3.51	3.78	4.55	7.68	8.36	3.80	0.30
Vmax/Km	1.05	0.50	0.70	0.913	1.31	0.79	0.24	0.89	0.18

NA - not applicable

Averages do not include BEW (male and female human pool).

[1] - The first average is obtained by averaging the values for each individual human donor sample.

[2] - The second AVG does not include sample EQB.

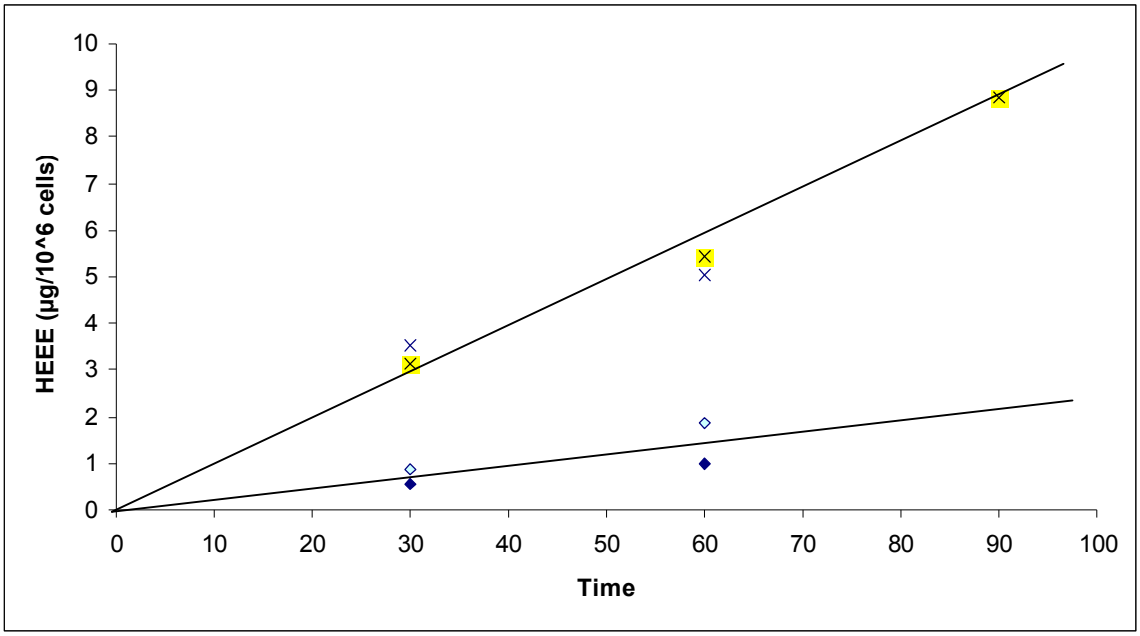


Figure 1. Production of HEAA in cryopreserved human hepatocytes from a pool of human donors incubated for 30-90 minutes with 0.5 or 25 mg/ml dioxane at a target cell density of 2.5×10^6 cells/ml in 250 µl. Data points represent duplicate incubations of individual preparations.

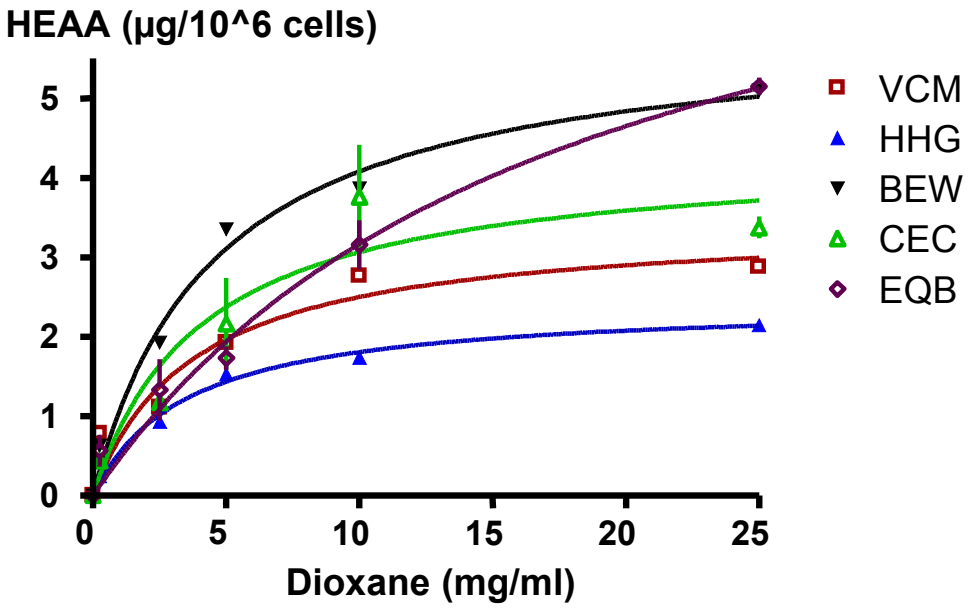


Figure 2. Production of HEAA in cryopreserved isolated hepatocytes from human donors incubated with initial concentrations of dioxane from 0.25 to 25 mg/ml. Symbols represent the average of duplicate incubations for each initial concentration of dioxane, error lines represent the range in the duplicates, symbols lacking lines indicate duplicates overlap. The lines represent the fit of the data to the Michaelis-Menten equation using Graphpad Prism.

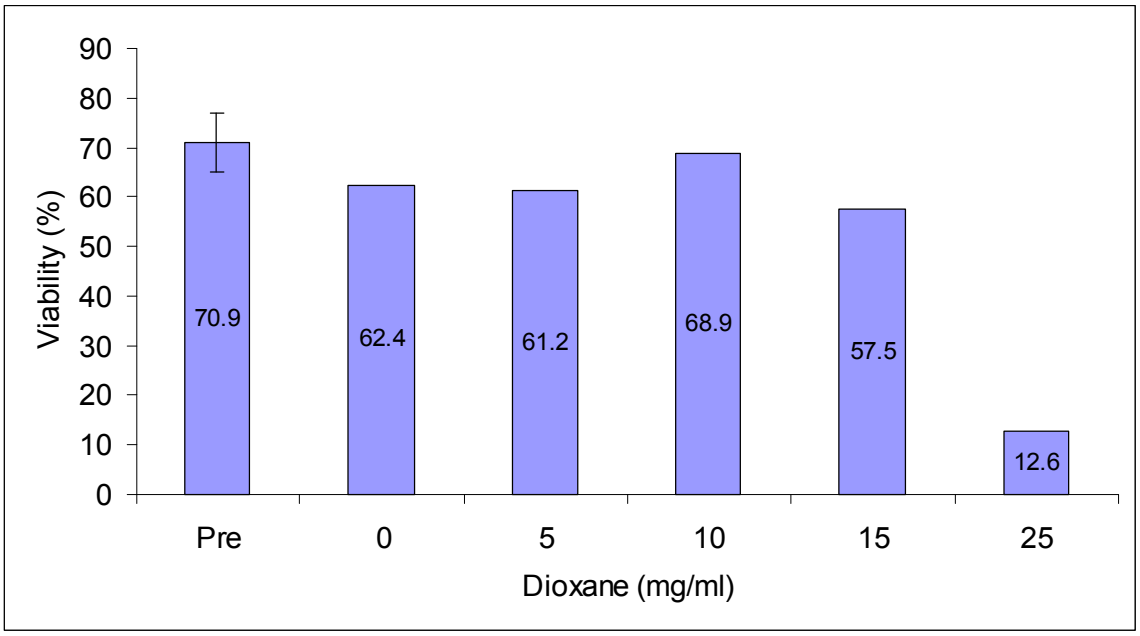
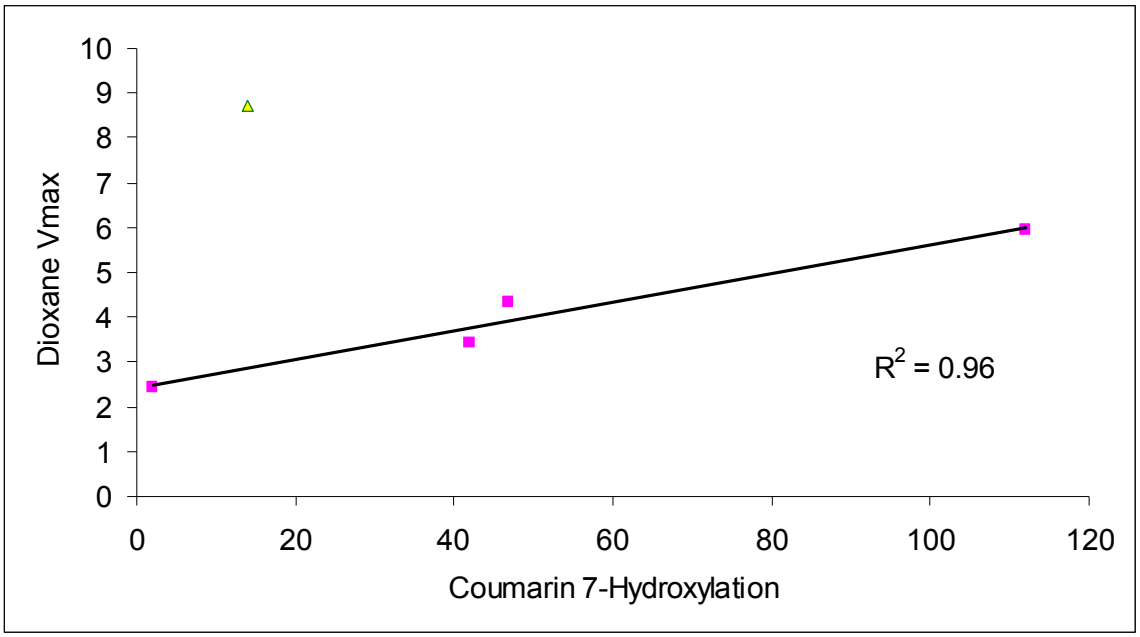


Figure 3. Trypan Blue Dye Exclusion as a measure of cell viability in human hepatocytes before (pre: n=4 different hepatocytes preparations from individual human donors) and after incubation for 1 hr with 0-25 mg/ml dioxane (n=1 or 2). The viability calculated during this study was consistently 10-20% lower than reported by the supplier (see table 1).



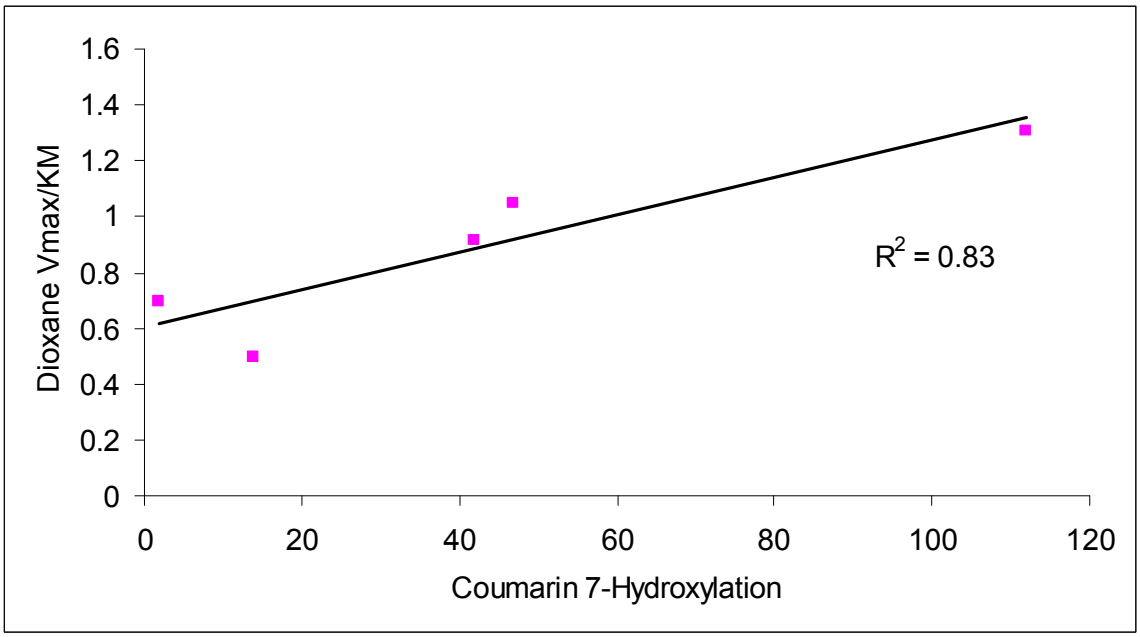


Figure 4. Correlation between supplier-provided rate of coumarin 7-hydroxylation (pmol/10^6 cells/min) and measured metabolism of dioxane. Top, Vmax; Bottom, Pseudo-first order rate of metabolism (V_{max}/K_m: μg×ml/hr×10^6 cells). In the top graph, the V_{max} for EQB (triangle) is not included in the linear regression.

APPENDIX

Table

Table A. Cellular Incubation Conditions

Human Hepatocytes	Target Cell Density (cells/ml)	Measured Cell Density (cells/ml)[1]	Incubation Volume (ml)	Cells/Incubation
CEC	2.5×10^6	1.62×10^6	0.25	4.04×10^5
EQB	2.5×10^6	1.69×10^6	0.25	4.24×10^5
HHG	2.5×10^6	1.89×10^6	0.25	4.72×10^5
VCM	2.5×10^6	2.57×10^6	0.25	7.15×10^5
BEW	2.5×10^6	3.60×10^6	0.25	8.99×10^5

[1] Cells were counted using trypan blue dye and a hemocytomer. Cell density is the number of live cells/incubation.

Table B. HEAA Concentrations in Individual Incubations after 60 Minutes

Rat Hepatocytes					
	HEAA (µg/ml)				
Dioxane (mg/ml)	Lot #CEC	Lot # EQB	Lot # HHG	Lot # VCM	Lot # BEW
0	0.0	0.0	0.0	0.0	0.0
0.25	0.71	0.96	0.64	2.02	2.25
2.5	1.90	2.26	2.38	4.95	6.92
5	3.49	2.52	3.94	7.12	12.06
10	5.43	5.35	6.11	7.41	13.91
25	5.29	8.73	5.51	2.86	18.33
50	6.49	8.06	4.60	2.78	15.81
	HEAA (µg/10^6 cells)				
Dioxane (mg/ml)	Lot #CEC	Lot # EQB	Lot # HHG	Lot # VCM	Lot # BEW
0	0.0	0.0	0.0	0.0	0.0
0.25	0.44	0.57	0.25	0.78	0.63
2.5	1.18	1.33	0.93	1.11	1.93
5	2.16	1.73	1.53	1.92	3.35
10	3.76	3.16	1.74	2.77	3.87
25	3.37	5.15	2.14	2.88	5.10
50	4.01	4.75	1.79	2.54	4.40

Note - the limit of detection was 0.25 µg/ml, however, values given in this table are after a no-metabolism (pre-boiled) background subtraction of approximately 0.4 µg/ml and are therefore above the limit of detection.
Note 2 - the data from the 50 mg/ml dioxane incubations was not used to calculate Vmax or Km due to potential toxicity (See Figure 1).